EFFECT OF ARECA NUT ON CULTURED HUMAN BUCCCAL FIBROBLAST: A CELL CULTURE STUDY

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CERTIFICATE

This is to certify that this dissertation titled **"EFFECT OF ARECA NUT ON CULTURED HUMAN BUCCAL FIBROBLAST : A CELL CULTURE STUDY"** is a bonafide record of work done by **YAKOB MARTIN** under our guidance during his postgraduate study period between 2007-2010.

This dissertation is submitted to **THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY,** in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY** in **ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI**. It has not been submitted (partial or full) for the award of any other degree or diploma.

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Betel quid (BQ) chewing is a popular habit in Taiwan, India and many Southeast Asian countries [1]. It has been estimated that, worldwide, around 600 million people chew areca nut [2]. Areca nut popularly known as betel nut accounts for a major portion, by weight of the betel quid, which is composed of betel leaf, areca nut, catechu and lime to which tobacco may or may not be added [3]. Condiments, sweetening agents and spices maybe added according to individual preferences [4].

Betel leaf is perishable and BQ preparation is complex, and this led to the emergence of commercial areca nut products about three decades ago. In India, areca nut is chewed as such or in the form of commercial products such as scented supari, manipuri tobacco, pan masala (areca quid, catechu, lime, flavors and spice), gutka or mawa (areca quid and tobacco) [4,5].

Oral submucous fibrosis is a pre-malignant and crippling condition of the oral mucosa characterized by submucosal fibrosis leading to atrophic changes in the epithelium resulting in mucosa appearing pale or whitish, presence of palpable fibrous bands, sensitivity to hot and spicy food and restricted mouth opening[6].

Oral submucous fibrosis was first described by Schwartz in 1952 among five East African women of Indian origin. This was followed by first description of this condition in India in 1953 by Lal and Joshi [5].

The overall prevalence of OSF has increased from 2.42 in 2002 to 6.42 per 1000 per year in 2004[7]. Rapid onset of the disease in younger chewers has been reported in a hospital based case control study in Chennai, South India[8].

Several studies concerning the etiologic factors of OSF have been done but not many focusing on the habitual use of different forms of areca nut. Areca nut contains several alkaloids of which arecoline is the most abundant. Many of the undesirable effects of areca nut have been attributed to arecoline because of its mutagenic activity in mammalian cells. Arecoline is also a cytotoxic agent to primary human buccal fibroblast cultures. Paradoxically, it has been experimentally observed that arecoline in lower concentrations stimulates collagen synthesis by fibroblasts *in vitro* [4, 5].

Strong association between areca nut use and OSF has been studied but not many have been done to assess the effects of alkaloids derived from products at a cellular level. Hence, the present study was done to isolate, culture and characterize fibroblasts from buccal mucosa of subjects without areca nut chewing habit and to evaluate the changes in the cultured fibroblast cells when exposed to extracts of various commercial areca nut products.

Aims and Objectives

I. To isolate and culture fibroblasts from the buccal mucosa of subjects who do not chew areca nut and have clinically normal appearing oral mucosa.

II. To evaluate the effects of commonly used areca nut products on the cultured fibroblast cells.

III. To evaluate and compare the following features

- The ratios between F_1, F_2, F_3 subpopulations.
- The ratios between mitotic and post-mitotic phenotypes.
- The growth curve and its derivatives for each cell line. (Seeding efficiency and population doubling time).

Hypothesis

Areca nut products can alter the phenotypic characteristics of buccal mucosal fibroblasts.

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History

The practice of betel quid or areca nut chewing dates back several thousand years. A ceremonial gift of dried tobacco leaves given to Columbus by native Americans in 1492 led to the introduction of tobacco into the rest of the world. It arrived in India in the 16th century when a sample of dried tobacco was presented to Emperor Akbar who patronized smoking. BQ chewing was already a socially well accepted practice and the introduction of tobacco reinforced this practice. An attempt to ban the rapidly spread habit of smoking in 1619 had little effect as the revenue from tobacco was already considerable[4].

The preparation of BQ varies in different countries. In India the BQ generally contains areca nut, lime, tobacco with or without piper betel leaf (PBL) whereas a piece of piper betel inflorescence is used instead of tobacco in the typical BQ preparation in Taiwan and Papua New Guinea[2].

Betel leaf being perishable and the preparation of BQ being complex, resulted in the evolution of commercial substitutes like *pan masala* and *gutka* about three decades ago. The products were made available in the market in 1985 as 4gm sachets. The website (www.newindia.com/kothari/) of the first major manufacturer of *pan masala* and *gutka* presented their strategy as '….to prepare convenient, anytime, anywhere substitute for pan….give some respectability to a habit that was considered low in image by genteel'[4].

Sushruta- the ancient Indian medical text- describes a condition termed 'VIDARI' under mouth and throat diseases as 'progressive narrowing of mouth, depigmentation of oral mucosa and pain on taking food' which are the typical characteristic features of OSF [6]. In 1952, Schwartz described five Indian from Kenya with a condition of oral mucosa including the palate and the pillars of the fauces, which he called "atrophia idiopathica (tropica) mucosae oris". Other names are "diffuse oral submucous fibrosis", "idiopathic scleroderma of mouth", "idiopathic palatal fibrosis", "sclerosing stomatitis" and "juxta-epithelial fibrosis". Later it was termed "oral submucous fibrosis (OSF)"[14].

The malignant potential of this condition was first described by Paymaster[15]. The first description of this condition in India was in 1953 by Lal and Joshi. The first report among non-Indians was from Taiwan by Su in 1954[5]. A strong association between areca nut use and oral submucous fibrosis has been established [10].

Only sporadic cases of OSF were reported during 1960's and 1970's.In 1980 Pindborg found that there were 250,000 OSF cases in India. In 1993 Phatak AG estimated the OSF cases in India to be two million. This is an eight-fold increase in the oral submucous fibrosis cases in less than 15 years[6].

Epidemiology

Shah N and Sharma P.P (1998) performed a case control study on 236 patients who belonged to New Delhi, to identify the relationship of various chewing and smoking habits to the development of OSF. They found that maximum number of OSF cases belonged to the age group of 21 to 30 years, with a male to female ratio of 2.2:1. Also, in the younger age group of 11-20 years, males were found to be more affected, the proportion being 6.5:1. It was also seen that 75% of the pan masala chewers took 4.6 years to develop the disease while quid chewers took 9.6 years to develop the disease. The overall effect of pan masala chewing when compared to quid chewing clearly showed that pan masala chewers developed the disease in almost half the time taken by nut/quid chewers. The relative risk to develop the disease was found to increase with the frequency of chewing rather than the duration of chewing. The role of tobacco in the development of the disease was not significant.[6]

Ranganathan K, Uma Devi M, Joshua E, Kriankumar K, Saraswathi T R (2004) carried out a case control study among 185 OSF patients in Chennai. Areca nut and pan masala were found to be more widely used than betel quid. More males used pan masala than females. Eight percent of the controls had chewing habits but their mean duration of usage was 3 years as compared to 6 years of the OSF cases. The highest risk of developing disease was for pan masala chewers followed by pan masala and alcohol users. When compared to them, betel quid chewers were found to have a reduced risk. Tobacco smoking and use of alcohol without use of areca nut products was not associated with OSF. The risk of developing OSF was double for patient below 21, who developed the disease within 3.5 years of having the habit compared to

patients of age 21 to 40 years who took 6.5 years of chewing habit to develop the disease.[8]

Yang Y.H, Lein Y-C, Ho P-S, Chen C.H, Chang J.S.F, Cheng T.C and Sheih T.Y (2005) conducted a study to investigate the risk of areca /betel quid chewing with and without cigarette smoking on OSF patients. A stratified case-control study was designed in the Taiwanese population in which they selected 102 patients with oral mucosal lesions of which 62 were diagnosed of having OSF. The areca/betel quid consumed in Taiwan has never contained tobacco. OSF was the most prevalent lesion in every combination of areca betel quid chewing and smoking habits (60.8%) followed by hyperkeratosis (39.2%) which was prevalent in chewing without smoking group. Cases of oral squamous cell carcinomas were seen in the quid only group. When 62 OSF patients were analyzed, people with smoking and areca nut chewing had a statistically significant odds ratio of 8.68 whereas in people with areca nut chewing and no smoking the odds ratio was 4.51.[16]

Pei-Shan HO, Yi-Hesin Yang, Tein-Yu Shieh, I-Yueh, Yun-Kwan Chen, Ko-Ni Lin et al (2006) carried out a case control study to identify the effects of areca nut, smoking and alcohol consumption on the coexistence of OSF and oral cancer. 104 histologically confirmed male OSF subjects were included, which consisted of 65 OSF subjects without oral cancer (control group) and 39 OSF subjects with oral cancer (case group). Age and consumption of alcoholic beverages showed a significant effect and the odds ratio were 1.07 in age and 1.5 in alcohol consumption. It was found that older age and increase in consumption of alcohol might be important malignant risk factors for oral cancer and OSF. Alcohol consumption was

significantly higher in patients having OSF with squamous cell carcinoma when compared to patients with OSF alone or along with the use of tobacco. Using regression models it was found that age and use of alcohol had a significant effect on malignant transformation of OSF. [17]

Hazarey V.K, Erlewad D.M, Mundhe K.A and Ughade S.N (2007) conducted a cross-sectional study to correlate the gender specificity for the different habits and severity of OSF. OSF occurred in younger men when compared to women, with the male to female ratio for occurrence of OSF being 4.9:1. Exclusive areca chewing was more prevalent with females. 77.8% of the OSF patients had multiple habits while 20.5% had exclusive areca chewing habit. The associated lesions with OSF were leukoplakia (4.8%), lichen planus (0.7%), erythroplakia (0.2%), betel chewers mucosa (0.7%) and squamous cell carcinoma (3.3%). [7]

Hsue Shue-Shue, Wang Wen-Chen, Chen Chung-Ho, Lin Cheng-Chung, Chen Yun-Kan and Lin Min-Lin (2007) carried out a follow up study to estimate the rate and time of transformation for various potentially malignant oral mucosal lesions in a Taiwanese population. The follow up time was defined as the duration between the onset of the initial diagnosis and the occurrence of confirmed oral cancer. A total of 1458 patients with histological diagnosis of various potentially malignant oral mucosal disorders were followed up for a period from 1991 to 2001. Amongst these 1458 patients with various potentially malignant oral epithelial lesions, 44 patients were found to progress to oral carcinoma proper giving an overall transformation rate of 3.02% and a mean time for transformation of 42.6 months. It was also found that

there was a malignant transformation rate of 1.9 % with a median duration of 52.3 months among the OSF patients. [18]

Etiology

OSF is a precancerous condition with a high risk for cancer development. The use of chilies *(Capsicum annum and Capsicum frutescens)* was suggested as possible etiological factors but currently the habit of areca nut chewing is recognized as the most important etiologic agent in the pathogenesis of this condition [19].

Several epidemiological studies have shown that areca nut is the etiological factor for oral submucous fibrosis[20]. A definite dose dependent relationship between areca nut consumption and causation of the disease exist. Frequency and duration of areca nut use are important in the disease causation [8].

Areca nut:

Areca nut (often referred as betel nut) is the endosperm of the fruit of the Areca catechu tree. The areca palm is native to South Asia and Pacific islands. The fruit is orange-yellow in colour when ripe. The fibrous pericarp is removed from the seed or endosperm, which then used fresh, after sun-drying or in a cured form.

Areca nut contains tannins (11.4-26%) of which gallotannic acid (18.03%) and D-Catechol (0.4%) are important. It contains several alkaloids (0.15-0.67%) like arecoline and arecaidine, of which arecoline is the most abundant[1, 2, 5,21.]

The pharmacologic effects of areca nut include addiction, euphoria, excessive salivation and tremor, attributable to the parasympathomimetic action of arecoline, the major alkaloid of areca nut [22].

Various areca nut preparations used in the Indian subcontinent are[7]

a) Supari – Areca nut alone
b) Betel quid- Areca nut, tobacco, betel leaf, slaked lime.
c) Mawa -Thin shavings of areca nut with some sun dried tobacco and slaked lime.
d) Gutka – Areca nut, powdered tobacco, catechu, condiments and slaked lime.
e) Panmasala – Areca nut, catechu, condiments and slaked lime.

Pathogenesis

The pathogenesis of OSF is believed to be multifactorial, triggering the disease by causing juxtaepithelial inflammatory reactions resulting in fibrosis of the oral mucosa. The fibrosis could be due to abnormal collagen, increased collagen production or decreased collagen destruction. Overall increased production and decreased degradation of collagen results in increased collagen deposition on the oral tissues, leading to fibrosis. This phenomenon is due to up regulation or down regulation of various pathways involved in the collagen turnover. The fibrosis of the sub-epithelial connective tissue accounts for the various clinical features found in OSF.

Initial events

When the betel quid is placed in the buccal vestibule for 15 minutes to one hour and repeated five to six times, alkaloids and flavonoids from the quid are absorbed into the oral mucosa and are metabolized facilitated by constant contact between the mixture and the oral tissues. These constituents along with the physical micro trauma produced by the areca nut leads to a juxta-epithelial inflammatory cell infiltrate. Over a period of time due to the persistence of the habit, chronic inflammation sets in followed by atrophy and ulceration of the mucosa. [23]

The inflammatory cells within the lesional tissue consist mainly of T lymphocytes with a high CD4:CD8 ratio. Studies have shown an increase in pro-fibrotic cytokines like IL-6 IL-1, TGF-β, PDGF at lesional site of OSF and an accompanied down regulation of anti-fibrotic cytokines like IFN-γ. The role of anti-fibrotic has been proved by studies which showed that local injections of IFN-γ reduce the contracture formation and facilitate mouth-opening. The pro-fibrotic cytokines produced by these inflammatory cells can create an environment favoring fibrosis.[24]

Increased collagen synthesis

Fibroblast proliferation

Studies have shown that areca nut causes fibroblast proliferation. OSF fibroblasts were found to have a doubling time of 3.2 days while for fibroblasts from normal mucosa it was 3.5 days. [25]

Increased collagen synthesis

Collagen is the major structural component of the connective tissue and its composition needs to be maintained for proper tissue integrity. Three main events are modulated by TGF-β that favours collagen production[23]:

a) Activation of procollagen genes: COL1A2, COL3A1, COL6A1, COL6A3 and COL7A1
b) Elevation of procolagen proteinase levels: PCP and PNP
c) Up-regulation of LOX

OSF fibroblasts show a 1.5 fold increase in the collagen production. Both normal and OSF fibroblasts produced 85% of type I and 15% of type III collagen.

The ratio of α 1(I) pro-collagen to α 2(I) pro- collagen was 3:1 in OSF in contrast to 2:1 as in normal. There is an over expression of pro-collagen mRNAs corresponding to the increased collagen production OSF. [25]

Morphologically, fibroblasts were classified into three types: F1, F2 and F3. OSF fibroblasts showed an increase in the F3 population of fibroblasts when compared to F1 population, but normal mucosa had greater number of F1 than F3 fibroblasts. F3 fibroblasts are large stellate shaped fibroblasts which are less proliferative and more collagen producing while F1 fibroblasts are spindle shaped, proliferative and less collagen producing. F2 fibroblasts are intermediate cells which are epitheloid in shape and are less proliferative than F1.[10]

Reduced Collagen destruction

Reduced fibroblast phagocytosis

OSF fibroblasts exhibited a 40% reduction in the collagen phagocytic cells and 48% reduction in the fibronectin phagocytic cells. Interestingly normal fibroblasts also when exposed to areca alkaloids showed a dose dependent reduction in phagocytic abilities.[26]

The two main events modulated by TGF-β which decreases the collagen degradation are[23]:

a) Activation of tissue inhibitor of matrix metalloproteinasegene (TIMPs)
b) Activation of plasminogen activator inhibitor (PAI) gene

Clinical features

Various investigators have correlated the salient clinical features and histological features. The onset is insidious over a period of 2-5 years. OSF patients usually present with rigidity or inability to open the mouth and burning sensation on taking hot and spicy food. Appearance of blisters or vesicles, ulcers, petechiae, melanosis and xerostomia are the usual initial symptoms. The frequently affected sites are buccal mucosa, retromolar region, and soft palate. As the disease progresses, the mucosa becomes blanched along with presence of palpable fibrous bands. Sub-mucosal fibrotic bands are palpable in buccal mucosa, labial mucosa, retromolar region and soft palate. Fibrosis of the soft palate can cause fixation, shortening and deviation of uvula. When tongue is involved it becomes immobile and depapillated. The dense fibrosis of the tissue around the pterygomandibular raphe is responsible for the varying degrees of trismus.

Fibrosis may extend into the pharynx and pyriform fossae causing referred pain in the ear.[14, 27]

Fibroblast

They are the predominant cells in the fibrous connective tissue. These cells are spindle shaped with oval nuclei having one or two minute nucleoli, eosinophilic to basophilic cytoplasm depending on the rate of synthetic activity. Fibroblasts are responsible for intracellular assembly of various extra cellular fibrillary and non fibrillary products such as, procollagen, pro-elastin and glycosaminoglycans.

Fibroblast growth characteristics

Hayflick L and Moorhead P.S (1961) isolated and characterized human diploid fibroblasts from fetal tissue and the cultured fibroblasts were subjected to chromosomal analysis to determine the polyploidy or diploid class of cells. Cultured diploid fibroblasts were seen to have three phases of growth. In the early growth phase or phase I, the cells freed from the tissue established themselves on the culture plate. This phase lasts for 1or 3 weeks and ends with the formation of a confluent plate. After the first subculture, it enters into phase II which is characterized by rapid cell multiplication. This phase lasts for 2-10 months. By the end of this time cell degeneration starts to take place. This is followed by the cell entering the phase III. This phase is characterized by less mitotic activity and increase in cellular degeneration.

They noted that there exists a finite limit for the cultivatable period for the human diploid fibroblast, which is approximately 50 subcultures or 2^{50} cell generations.

They also implanted diploid fibroblasts into hamster check pouches and homotransplanted the same on to terminal cancer patients. None of the transplanted

diploid cells were able to produce any evidence of growth while transformed HeLa cell lines inoculated in the same manner, grew.[28]

Todaro G.J and Green H (1963) conducted a study where they disaggregated mouse embryo fibroblast cells and grew it in monolayers and were cultured at concentrations 3 x 10^5 and 6 x10^5 cells per plate with a doubling time of 60 hours and 30 hours respectively. Between the 10^{th} -20^{th} generation, the growth of the cells declined and doubling time exceeded 70 hours. From the 15^{th} -30^{th} generation, the growth rate of cells rose again and reached to a value similar to the beginning. The culture was then considered established.

This culture became tetraploid in later stages. The cells had the ability to grow in low concentration density ($5x10^4$) and showed a progressive increase in the saturation density. Cell morphology of early established lines was found to be similar to normal. The cells were able to form multilayers, in crowded cultures and these cells were shown to grow for 100 generations raising growth rate. They did not show any indication of growing out.[29]

Schneider E.L and Mitsui Y (1976) conducted a study to evaluate the difference in the *in vitro* life span of fibroblast cultures derived from elderly (63-92 years) and younger (21-36 years) individuals. A decrease was found in the rate of migration, culture senescence and replicative capacity in the fibroblasts derived from older aged subjects. This decrease was found to be statistically significant. The percentage of replicating cells and saturation density were found to be 50% higher in fibroblasts derived from younger donors, but there was no statistically significant difference in

the seeding efficiency, cell volume or macromolecular contents in the fibroblasts derived from the two age groups.[30]

Angello J.C, Pendergrass W.A, Norwood T.H and Prothero J (1989) performed a study to assess the increase in fibroblast cell size when cultured in low serum and low density conditions. For this, they cultured human diploid fibroblast (HDLF) like cells and chick embryo fibroblasts. It was observed that larger sized fibroblasts had lower replicative capacity. Cells cultured in low serum (0.1%) and high density produced smaller cells retained their potential to replicate. The large and small G1 cells were sorted and cloned. The small cells produced 10 progeny while the larger cells produced 4.3 progeny. These results helped to conclude that the ability of fibroblasts to replicate is directly proportional to cell size.[31]

Role of Areca nut in collagen production and the development of Submucous fibrosis

Van Wyk C.W,Oliver A, De Miranda C.M,Van der Bijil P,Grobler-Rabie A.F (1994) conducted a study to observe the effects of areca nut extracts on cultured oral mucosal fibroblasts. For this they exposed the fibroblast cells to three different forms of areca nut - aqueous extracts of raw, baked and boiled, at concentrations of 0, 50, 100, 150, 300 and 500μg/ml. The arecoline and arecaidine levels were also estimated using HPLC technique. They observed that at concentration as low as 50μg/ml, all extracts of areca nut inhibited oral fibroblast cell proliferation. At concentrations of 50-150μg/ml, cell numbers remained constant or showed limited proliferation. Total cell-death was observed at concentrations between
300-500μg/ml.[32]

Jeng J.H, Kuo M.L, Hahn L.J and Kuo M.Y.P (1994) studied the effects of
a) Betel nut alkaloid-arecoline, b) Betel quid constituents –betel nut (BN), inflorescence (IFN) and lime and c) Betel nut polyphenol –catechin, on cultured oral fibroblast cells. They observed that arecoline, catechin, extracts of betel nut and inflorescences were cytotoxic in a dose dependent manner, while lime at concentrations 500-800 μg/ml increased the cell proliferation. It was also found that betel nut (BN), inflorescence (IFN) extracts induced DNA break up. Inspite of arecoline and catechin being cytotoxic, they didn't produce any DNA break up. Among the antioxidants, glutathione and cystine had cyto-protective effect against areca nut induced cytotoxicity.[33]

Kuo M.Y.P, Chen H.M, Hahn L.J, Heish C.C and Chiang C.P (1995) studied collagen biosynthesis from OSF fibroblasts. OSF fibroblasts showed a 1.5 fold increase in the collagen production. Both normal and OSF fibroblasts produced 85% of type I and 15% of type III collagen. The ratio of pro collagen α 1(I) to pro collagen α 2(I) was 3:1 in OSF in contrast to 2:1 in normal. This presence of excess of pro collagen α 1(I) in OSF indicates the presence of collagen molecules composed entirely of pro collagen α 1(I) which is more resistant to degradation. This type of collagen accounts for 25% of the type I collagen produced by OSF fibroblasts. [34]

Van Wyk C.W,Oliver A, Helden E.G H,Grobler-Rabie A.F (1995) conducted a study to evaluate and compare the proliferation of oral fibroblasts and skin fibroblasts from OSF patients and normal controls. The fibroblast cells were obtained from fibrous bands present on the buccal mucosa and the inner aspect of the forearm of patients with OSF as well as from healthy non-areca nut chewing individuals. They observed at concentrations of 0.1-10.0μg/ml, the cell exposed to arecoline followed a normal proliferation pattern, except for the oral fibroblasts of OSF patients which were slightly depressed. Cell growth was inhibited at the concentration of 400μg/ml. the morphology of cells from both skin and buccal mucosa was comparable to the spindle-shaped F1 fibroblast, epithelioid FII cells. Spindle-shaped fibroblasts dominated in both skin and mouth cell-cultures on day 6.[13]

Ma R.H, Tsai C and Sheih T.Y (1995) compared the population doubling time of OSF fibroblasts with normal controls. The found that OSF fibroblasts had a doubling time of 3.2 days while for normal controls it was 3.5 days. They also observed that both protein content and lysyl oxidase activity was higher for OSF fibroblasts when

compared to normal fibroblasts. The immunoenzymatic assay for BrdUrd incorporation revealed that OSF fibroblasts proliferate significantly faster than NM fibroblasts under standard culture conditions. Both total protein content (10.84±1.15 mg/ml) and lysyl oxidase activity (3558.6±345.5 cpm/106 cell) in OSF fibroblasts were greater than in NM fibroblasts (6.35±0.96 mg/ml and 2436.0±352.6 cpm/10^6 cell).[25]

Jeng J.H, Lan W.H, Kuo M.L, Hahn L.J, Heish C.C and Kuo M.Y.P (1996) demonstrated the association of betel quid chewing and gingival health. The study was carried out by studying human gingival fibroblast (HGF) when exposed to arecoline. At concentrations higher than 0.4mM, inhibited cell attachments, cell spreading; cell migration in a dose dependent manner. It was considered to be due to the intracellular depletion of glutathione. 24 hour incubation of cells with 1 mM of arecoline left only 35% cells viable.[35]

De Waal J, Van Wyk C.W, Oliver A and Martiz J.S (1997) compared the morphology of fibroblasts cultured from healthy oral mucosa and mucosa of patients with OSF. They also collated the occurrence of cell types of similar morphology. OSF fibroblasts showed an increase in the F3 population when compared to F1 population following an 8 day observation period. They found that F3 fibroblasts which were large stellate shaped cells as compared to F1 fibroblasts which were spindle shaped cells. F2 fibroblasts were epitheloid cells having intermediate characters.[10]

Chang Y.C, Tai K.W, Cheng M.H, Chou L.S.S and Chou M.Y (1998) performed cytotoxicity and genotoxicity assays to investigate the pathobiological effects of arecoline on cultured human buccal fibroblasts. Arecoline increased polynucleic acids at a concentration of 0.1to 10 μg/ml in a dose dependent manner. At a concentration higher than 50μg/ml, arecoline was cytotoxic to the cultured fibroblasts. Genotoxicity was not observed even with concentrations as high as 400μg/ml of arecoline .On the other hand, glutathione (at 600 μg/ml) and glycyrrhizin (at 200 μg/ml) were found to have cyto-protective effects against arecoline. These results indicated that arecoline was a cytotoxic agent and not genotoxic to human fibroblasts. Increased consumption of glutathione and glycyrrhizin rich food was found to reduce the oral diseases associated with betel-quid chewing. [36]

Tsai C, Ma R.H and Sheih T.Y (1999) cultured fibroblasts from OSF and normal subjects. Collagen and fibronectin coated beads were incubated in normal and OSF fibroblast cultures for 16 hours to assess their phagocytic abilities. OSF fibroblasts exhibited a 40 % reduction in the collagen phagocytic cells and 48% reduction in the fibronectin phagocytic cells. Normal fibroblasts when exposed to areca alkaloids showed a dose dependent reduction in phagocytic abilities.[37]

Chang Y.C, Hu C.C, Tseng T.S, Tai K.W, Lii C.K and Chou M.Y (2001) demonstrated that arecoline at concentrations higher than 50μg/ml was cytotoxic to fibroblasts in a dose dependent manner. Also, it was seen that arecoline significantly decreased the GST activity, but it did not increase lipid peroxidation .the addition of extracellular nicotine acted synergistically on the arecoline-induced cytotoxicity. [22]

Trivedy C, Meghi S, Warnakalasuriya K.A.A.S, Johnson N.W, Harris (2001)

Incubated fibroblasts with 0.1 to 500μM of $CuCl_2$ .The fibroblasts showed an increased collagen production. There was no increased non collagenous protein production or cell proliferation.[20]

Sheih D.H, Sheih T.Y, Chiang L.C (2003) conducted a study to assess the cytoxic role of arecoline, arecaidine and safroline in betel-quid and to quantify the TIMP-1 expression. Both normal and OSF fibroblasts cells were cultured from the same patient. OSF fibroblasts produced more tissue inhibitor for matrixmetallo protease I (TIMP-1) protein than normal fibroblasts. They concluded that increased mRNA expression of TIMP-1 in buccal mucosal fibroblast by arecoline and safrole has a possible role in the pathogenesis of OSF.[38]

Sheih D.H, Chiang L.C, Lee C.H, Yang Y.H and Sheih T.Y (2004) carried out a study to investigate the effects of arecoline, safrole and nicotine on cultured fibroblasts obtained from tissue specimen from the buccal mucosa of OSF patients and healthy controls. They found that the phagocytic activity of OSF fibroblasts and normal fibroblasts were inhibited in a dose dependent manner on exposure to arecoline, safrole and nicotine.[26]

Tsai C.I, Yang S.F, Yang Y.J, Chu, IIseih Y.S and Chang Y.C (2004) studied m RNA expression of IL-6 in the cultured fibroblasts from OSF patients and buccal mucosal fibroblasts from normal controls (NBM). The IL-6 expression in OSF fibroblasts was found to be elevated. Fibroblasts from normal buccal mucosa showed a raised IL-6 expression when exposed to arecoline in a dose dependent manner. In

order to determine the role of intercellular glutathione (GSH) levels on IL-6 levels in NBM fibroblasts, NBM fibroblasts were pretreated with GSH elevating agent 2-Oxothiazolidine-4-Carboxylate (OZT) and a GSH reducing agent Buthione sulfoxamine (BSO) and was exposed to arecoline. The IL-6 levels were determined. In NBM fibroblasts arecoline induced 2.7 folds of IL-6 mRNA expression while BSO pretreated fibroblasts showed an increase in IL-6 activity by 3.4 folds. The cells pretreated with OZT showed only a marginal increase of 1.7 fold in IL-6 activity. [39]

Fibroblast heterogeneity

Castor *et al* in 1960 conducted the first study on fibroblast heterogeneity where they assessed the different fibroblasts cultures from dermis articular tissue, mesothelial surface & periostium. They found that mesothelial fibroblasts had a slower proliferation rate and produced more acid mucopolysacchride than other fibroblasts. Fibroblasts are not homogenous as they differ in their morphology and function because fibroblasts in different tissues and organs have varied function [40]

Mitsui Y and Schneider E.L (1976) considered human fetal lung fibroblasts (WI 168) cell lines which were in three different phases of culture to assess the cell replicative potential. The different phases observed were in the early passage (cells at 19 population doublings), middle passage (cells at 35 population doublings) and late passage (cells at 47 population doublings) based on cell size by gravity sedimentation.[^{3}H] TdR labeling was done to assess its replicative potential.

They observed that late passage cells yielded cell population enriched for large cell volume and slow replication. Fibroblast cells seen in the early and middle passages were small and had faster replicative potential. When re-introduced into

tissue culture conditions, cell populations lost their phase properties and resembled the un-fractionated cell culture at the same level of *in vitro* passage.[41]

Harper R.A, Grove G (1979) carried out fibroblast culture on human dermis to study their saturation level and population doubling time**.** Human dermis is divided histologically into two layers, upper papillary dermis and lower reticular dermis. It was found that papillary dermis was metabolically active and had more fibroblasts than reticular dermis. On culturing fibroblasts from papillary dermis and reticular dermis separately from the same patient; it was found that saturation level and population doubling time of papillary dermis fibroblasts were always higher than in reticular dermis fibroblasts.

Atrophy of the papillary layer of skin was observed with aging, so a full thickness skin culture will have more reticular fibroblasts than papillary fibroblasts resulting in less replicative short lived fibroblast cultures compared to cultures from tissues of younger individuals.[42]

Smith J.R, Whitney R.G (1980) cultured human embryonal lung fibroblasts *in vitro* and found that there was heterogeneity in the proliferative potential which appears rapidly within a single clone of cells.

Cultures from a single cell after 16 population divisions (PD) had cells with doubling potential ranging from 0-33 PDs whose distribution curve was bimodal. Two cells from the same mitotic event can differ by as many as 8 PDs.[43]

Mollenhauer J and Bayreuther K (1986) studied rat skin and lung fibroblasts and based on their morphological differences found three subpopulations F I, FII, F III.

FI cells are diploid, spindle shaped and highly proliferative. and synthesize low levels of type-I and -III collagen. FII cells are also diploid epithelioid shape, proliferate slowly, and produced more collagen as compared to FI cells. FIII fibroblasts are large, stellate, tetraploid cells that proliferate more slowly than the other types but synthesize large amounts of collagen. [11]

Somerman M.J, Archer S.Y, Imm G.R and Foster R.A (1988) evaluated periodontal ligament and gingival fibroblasts from the same patient for heterogeneity even though they were similar in morphology and proliferation rates.

Early passage periodontal ligament fibroblasts showed contact inhibition but not gingival fibroblasts. Periodontal ligament fibroblasts showed more protein production and alkaline phosphatase activity than gingival fibroblasts.[44]

Bayreuther K, Rodemann H.P ,Hommel R, Dittmann K, Albiez M and Francz P.I (1988) found that further differentiation occurred along the terminal cell lineage with mitotic fibroblasts, F-I, F-II, F-III. It was observed in culture studies that when the mitotic capacity of F-III is exhausted, they become post-mitotic and differentiate into cell-lineage sequences following the mitotic sequence as F-IV, F-V, F-VI and degenerating fibroblast F-VII. F-I is a small spindle shaped cell. F-II is a small epitheloid cell. F-III is a large pleomorphic epitheloid cell. F-IV is a large spindle shaped cell. F-V is a larger epitheloid cell. F-VI is the largest epitheloid cell of the fibroblast series and F-VII is the degenerating fibroblast.[12]

Kawase T, Sato S, Maike K and Saito S (1988) conducted a study to assess the alkaline phosphatase activity on different cells associated with the dentition. For this they studied alkaline phosphatase activity of human periodontal ligament fibroblast (HPLF), human alveolar bone cells (HABC) and gingival fibroblasts. They observed that alkaline phosphatase synthesized by HPLF were similar to HABC and that gingival fibroblasts had no alkaline phosphatase activity. They also observed that 1, 25 di (OH) cholicalciferol can markedly stimulate alkaline phosphatase activity in HPLF and HABC and also that it had no effect on gingival fibroblasts. As HPLF's alkaline phosphatase activity is similar to HABC, HPLF can also be termed as osteoblastic fibroblasts. [45]

Oslen D.R, Peltonen J, Jaakkola S, Chu M.l and Uttio J (1989) carried out a study on human skin fibroblasts to assess their ability to synthesize genetically distinct procollagens. Their study revealed the presence of mRNA transcripts for collagen types I, III, IV, V and VI but not for collagen II. It was also observed that mRNA expression for type I and VI was highest and for type V and IV lowest.

The homogeneity of skin fibroblasts with regard to procollagen gene expression was shown as the individual fibroblasts were found to be capable of expressing multiple procollagen genes. [46]

Breen E, Falco V.M , Absher M and Cutroneo K.R (1990) conducted experiments where they isolated different types of fibroblasts depending upon the type of collagen coating on the cell surface. They were able to derive two sub populations from the Human Lung Fibroblasts by means of Fluorescent Assisted Cell Sorting (FACS). They identified that one of the sub populations had cells with type I collagen surface

coating in association with more of type I procollagen mRNA while the other sub population had cells with type III collagen surface coating in association with type III procollagen mRNAs. These fibroblast subgroups were able to maintain their phenotype even after four subcultures. [47]

Goldring S.R, Stephenson M.L, Downie E, Krane S.M, and Korne J.H (1990) studied the fibroblast heterogeneity in matrix biosynthesis and response to parathormone and prostaglandin E2. Fibroblasts were cultured from three skin samples and 24 cloned sub strains were made.

Sub strains from two parent samples were shown to be responsive to prostaglandin E2 and parathormone indicated by an increase in the C-AMP levels. The third parent culture was responsive to parathormone but none of its clones were responsive but all clones were responsive to prostaglandin E2.

All sub strains produced type I and type III collagen with variation in the amounts. Correlation was found between steady state levels of α 1(I) pro collagen mRNA and incorporation of [^{3}H] proline into α 1(I) pro collagen chains. Levels of type I and type III pro collagen mRNAs showed positive correlation indicating a similar coordinate regulation of type I and III collagen gene expression.[48]

Sempowski G.D, **Derdak S and Phipps R.P (1996)** carried out tests to evaluate the collagen production from fibroblasts when subjected to different environments. They found that IL-4 induced 100% increase in the collagen production in Thy-1$^+$ fibroblasts while no effect was there on Thy-1$^-$ fibroblasts collagen production. The collagen production was reduced by 50% in both Thy-1$^+$ and Thy-1$^-$ in presence of IFN-γ. A slight increase in the collagenase activity was seen in Thy-1$^+$ fibroblasts on

exposure to IFN-γ but not present in Thy-1$^-$ fibroblasts. There was increased expression of Tissue inhibitor of matrixmetallo protease (TIMP) in both subtypes more in Thy-1$^-$ fibroblasts on exposure to IL-4 or IFN-γ.[49]

Sorrel J .M, Baber M.A, Caplan A.I (2006) demonstrated a study to assess fibroblast heterogeneity within a region of dermis (epidermis and papillary dermis) for which skin at a depth of 40μm was selected. Cells released by enzymatic digestion were cloned by limited dilution. Initial selection was based on morphotype and proliferation pattern of the cells. These clones of cells isolated by limited dilution were found to be different in their rate of collagen lattice contraction, ability to organize fibronectin matrix and release of specific growth factors and interleukins. [50]

High Performance Liquid Chromatography in analysis of the Areca nut products:

Chromatography is the term used to describe a separation technique in which a mobile phase carrying a mixture is made to move in contact with a selectively absorbent stationary phase. The mobile phase is a solvent which is pumped under high pressure through a column. The stationary phase is a finely divided solid held inside the column. Different components of the sample are carried forward at different rates by the moving liquid phase, due to their differing interactions with the stationary and mobile phases. There are a number of different kinds of chromatography, which differ in the mobile and the stationary phase used.

HPLC is a chemistry based tool for quantifying and analyzing specific chemical compounds within the mixture. The sample is dissolved in a solvent (like water or alcohol) and thus the term liquid chromatography. A detector measures response changes between the solvent itself and the solvent and sample when passing through it. The electrical response is digitized and sent to a data system.

Simona Pichini, Manuela Pellegrini, Roberta Pacifici, Emilia Marchei, Janeth Murillo, Carme Puig, Oriol Vall and Oscar Garcı´a-Algar (2003) carried out a study to assess the eventual fetal exposition to arecoline by the analysis of neonatal biological matrices. A high performance liquid chromatography (HPLC) method with mass spectrometric detection was described for determination of arecoline in newborn meconium, urine and cord serum using pilocarpine as internal control. The samples were analyzed by HPLC coupled to an electrospray (ESI) interface and a quadrupole mass spectrometer. Chromatography was performed on a

C8 reversed-phase column using 10mM ammonium acetate (pH 4.3)/acetonitrile (90:10, v/v) as mobile phase. The method was validated over the concentration range 0.))%-1.00µg/g meconium, 0.004-1.00µg/mL cord serum and 0.001-1.00µg/mL urine. The mean recoveries ranged between 86.5 and 90.7% for arecoline in different biologic matrices, with precision better than 10%.[51]

Stephen Cox, Irina Piatkov, E.Russell Vickers, Gary Ma (2004) conducted a study employing HPLC method for determination of arecoline in saliva, using arecaidine as internal control. The optimal ultraviolet wavelength was shown to be 215nm for detecting arecoline in the mobile phase. Arecoline was extracted from saliva with hexane-isoamyl alcohol (1%) and reconstituted with mobile phase for HPLC. They proved that HPLC is a reliable, cost-effective and efficient method for the determination of arecoline concentration in saliva.[52]

Data analysis was done using SPSS (Statistical package for social science) version 14.

Linear regression analysis was performed

- To derive the slope from growth curves of each cell line.

Correlation coefficients were determined to analyze

- The F1, F2 and F3 ratios in each cell line and between each group.
- Mitotic cell to post mitotic cell ratios in each cell line and between each group.

This study was carried out to assess the phenotypic and proliferative alterations in the cultured fibroblasts obtained from the buccal mucosa of patients without any chewing habits when exposed to a commercially available areca nut product extract.

Buccal mucosal fibroblast cell lines were established from six subjects. The subject inclusion criteria were subjects who do not have the habit of chewing areca nut and have clinically normal appearing oral mucosa, i.e., *n=3* (Control group) and *n=3* (Study group).

The clinical history and cell line designations of the study subjects are given in (Table1). Buccal mucosal fibroblasts from the subjects were grown in complete media, on reaching confluency they were subcultured. In the study group, the fibroblast cells were exposed to areca nut extracts while the control group was cultured without exposing them to any areca nut forms.

Growth curve was plotted for the control group and population doubling time and seeding efficiency was derived from the slope to characterize the normal buccal fibroblast. (Table 5, 9, 13).

Extraction yield from Areca nut product:

80gm of a commonly used commercial areca nut product was subjected to hydro-alcoholic extraction and a yield of 21% was obtained. (Table 2).

HPLC analysis of arecoline and arecaidine in reference:

Samples of arecoline (Sigma ^{TM}) and arecaidine (Sigma ^{TM}) were subjected to HPLC and the concentration level of arecoline was 20.538µg/ml and the concentration of arecaidine was 15.932µg/ml. (Table 3).

HPLC analysis of arecoline and arecaidine in extract of areca nut product:

The extract obtained, after hydro-alcoholic extraction of the areca nut product, was subjected to HPLC and the concentrations of arecoline was 11.638µg/ml and arecaidine concentration was 761.808µg/ml. (Table 4).

GROWTH CURVE, F1:F2:F3 FIBROBLAST SUBPOPULATION RATIO, MITOTIC: POST-MITOTIC FIBROBLAST SUBPOPULATION RATIO.

Control Group

1st Normal mucosa cell line (1 NB)

Growth curve and its derivatives (Graph 3, Table 5)

The initial seeding concentration was 12 $x10^3$ cells/ well/ ml. There was a loss of cells within 12 hours of attachment time. The seeding efficiency was 58.3 %. There was a gradual increase in the slope of the growth curve from day 3 to day 8. Population doubling time calculated from the slope of the graph was 43.08 hours. Plateau phase was reached by the 4^{th} day of the culture.

F1:F2:F3 Fibroblast subpopulation ratio (Graph 4, Table 6,7)

There was an increase in F2 ($p<0.05$) and F3 ($p>0.05$) subpopulation of fibroblast during an 8 day observation period. On the contrary, F1 subpopulation ($p<0.01$) showed a decrease during the observation period.

Mitotic: Post-mitotic fibroblast subpopulation ratio (Table 8, 28)

There was a decrease in the mitotic fibroblast subpopulation during an 8 day observation period in comparison with post-mitotic fibroblast subpopulation, and the change was statistically significant.

There was an increase in the post-mitotic fibroblast subpopulation during an 8 day observation period in comparison with mitotic fibroblast subpopulation and the change was statistically significant.

2nd Normal mucosa cell line (2 NB)

Growth curve and its derivatives (Graph 4, Table 9)

The initial seeding concentration was 12 $x10^3$ cells/ well/ ml. There was a loss of cells within 12 hours of attachment time. The seeding efficiency was 50%. There was an increase in the slope of the growth curve from day 1 to day 6. Population doubling time calculated from the slope of the graph was 36.12 hours.

F1:F2:F3 Fibroblast subpopulation ratio (Graph 5, Table 10, 11)

During the 8 day observation period, an increase in the fibroblast subpopulation of F2 ($p<0.000$) and F3 ($p>0.05$) was seen. Decrease in F1 fibroblast subpopulation ($p<0.000$) was also noted during this period.

Mitotic: Post-mitotic fibroblast subpopulation ratio (Table 12,28)

There was a decrease in the mitotic fibroblast subpopulation during an 8 day observation period in comparison with post-mitotic fibroblast subpopulation, and the change was statistically significant.

There was an increase in the post-mitotic fibroblast subpopulation during an 8 day observation period in comparison with mitotic fibroblast subpopulation and the change was statistically significant.

3rd Normal mucosa cell line (3 NB)

Growth curve and its derivatives (Graph 6, Table 13)

The initial seeding concentration was 12 $x10^3$ cells/ well/ ml. There was a loss of cells within 12 hours of attachment time .The seeding efficiency was 50 %. There was a steady increase in the slope of the growth curve from day 1 to day 7. Population doubling time calculated from the slope of the graph was 22.85 hours.

F1:F2:F3 Fibroblast subpopulation ratio (Graph 7, Table 14, 15)

An increase in the F3 subpopulation of fibroblast ($p<0.01$) during an 8 day observation period along with an increase in the F2 subpopulation of fibroblast ($p>0.05$) was seen. There was a decrease in the F1 subpopulation of fibroblast ($p<0.01$) during the observation period.

Mitotic: Post-mitotic fibroblast subpopulation ratio (Table 16,28)

There was a decrease in the mitotic fibroblast subpopulation during an 8 day observation period in comparison with post-mitotic fibroblast subpopulation but the change was not statistically significant.

There was an increase in the post-mitotic fibroblast subpopulation during an 8 day observation period in comparison with mitotic fibroblast subpopulation but the change was not statistically significant.

Study Group

4th Normal mucosa cell line (4 NB)

F1:F2:F3 Fibroblast subpopulation ratio (Graph 8, Table 17, 18)

There was an increase in the F3 subpopulation of fibroblast ($p<0.01$) during a 5 day observation period along with an increase in the F2 subpopulation of fibroblast ($p<0.05$) was seen. There was a decrease in the F1 subpopulation of fibroblast ($p<0.001$) during the observation period.

Mitotic: Post-mitotic fibroblast subpopulation ratio (Table 19,28)

There was no post mitotic fibroblast subpopulations observed during the 5 day observation period.

5th Normal mucosa cell line (5 NB)

F1:F2:F3 Fibroblast subpopulation ratio (Graph 9, Table 20, 21)

During the 5 day observation period, an increase in the F3 subpopulation of fibroblast ($p>0.01$) along with an increase in the F2 subpopulation of fibroblast ($p<0.05$) was seen. There was a decrease in the F1 subpopulation of fibroblast ($p<0.05$) during the observation period.

Mitotic: Post-mitotic fibroblast subpopulation ratio (Table 22,28)

There was no post mitotic fibroblast subpopulations observed during the 5 day observation period.

6th Normal mucosa cell line (6 NB)

F1:F2:F3 Fibroblast subpopulation ratio (Graph 10, Table 23, 24)

An increase in the F3 subpopulation of fibroblast ($p>0.01$) during the 5 day observation period along with an increase in the F2 subpopulation of fibroblast ($p>0.05$) was seen. There was a decrease in the F1 subpopulation of fibroblast ($p<0.01$) during the observation period.

Mitotic: Post-mitotic fibroblast subpopulation ratio (Table 25,28)

There was no post mitotic fibroblast subpopulations observed during the 5 day observation period.

Over the years, OSF, a crippling, irreversible disease of the oral mucosa, has gained importance among the dental professionals in different parts of the world. Various studies, epidemiological and case-reports have shown that the peculiarity of this disease is that it is confined to particular geographic regions, especially the Indian sub-continent and South Africa. These findings have led to the concept that dietary or cultural habits prevalent in these regions could act as the aetiological factor.

OSF is characterized by fibrosis of the submucosa.[53] On exposure to various alkaloids of areca nut, there is an accumulation of inflammatory cells at the submucosal region which selectively induce high collagen producing fibroblast phenotype.[54] Fibroblasts are a heterogeneous group of cells whose morphology and function varies depending on the local environment.[46] Exposing normal fibroblasts to various areca nut products in cultures or growing the altered fibroblast directly from the pathologic site and studying their characteristics is a valuable tool in our understanding of the disease.

In the last few decades, small and attractive sachets of betel quid substitutes have become widely available in India and are found to be very popular among the younger age group. These products are flavored and sweetened dry mixture of areca nut, catechu and slaked lime with tobacco *(gutka)* or without tobacco *(pan masala).* These products have been strongly implicated in the incidence of OSF, even with a short period of use. As *gutka* and *pan masala* are mixtures of several ingredients, the role players in the resulting disease are yet to be studied. Reviewing various literatures on the pathogenesis of OSF, we identified the alkaloids to play a major role in pathogenesis of OSF.

In our study we cultured fibroblasts from the buccal mucosa of patients without chewing habits or existing oral lesions to assess the effects of areca nut products on

them. 80gm of a commercial areca nut product was subjected to hydro-alcoholic extraction and the yield was 21%.(Table 2).In HPLC, retention peak areas and ultraviolet absorption spectra were used to identify and quantify arecoline and arecaidine in the areca nut product. The chromatography was performed using two different mediums. Deionized water was used because it is used in cell cultures medium in which the exposure was done. Phosphated buffer saline (PBS) was used as the other medium because it is similar to the human saliva and its pH was adjusted to HPLC optimal level of 7.8.[52]Arecaidine concentration was found to be 761.808µg/ml and arecoline concentration 11.638 µg/ml in phosphated buffer saline (PBS).(Table 4) In deionosed water the arecoline concentration was 3.490 µg/ml and arecadine was 716.736 µg/ml.

To assess the effects of areca nut product on cultured normal buccal fibroblast, three cell lines were exposed to the areca nut extract and compared with the non-exposed control cell-lines. The shifts in the fibroblast phenotypes were studied based on the subpopulation model used by de Waal J *et al* in 1997 [14] on OSF fibroblasts as F1,F2 and F3 and fibroblast subpopulation model developed on human skin fibroblasts by Bayreuther *et al* in 1988 as mitotic and post mitotic.[20]

The description of buccal mucosal fibroblast subpopulations as F1, F2, F3 were based on the fibroblast subpopulation description done by Mollenhauer and Bayreuther in 1986 on rat skin fibroblasts.[11] In 1988 Bayreuther *et al* described human skin fibroblasts.[12] They found that subpopulations of mitotic fibroblasts (F1, F2, and F3) during the course of growth exhaust their mitotic abilities and becomes post-mitotic phenotypes (F4, F5, F6 and degenerating fibroblast F7). F1 is a small spindle shaped cell. (Fig 12) F2 is small epitheloid. (Fig 13) F3 is large pleomorphic and epitheloid. (Fig 14) F4 is large spindle shaped. (Fig 15) F5 is larger epitheloid,

larger than F3. (Fig 16) F6 is the large epitheloid, larger than F5. (Fig 17) and F7 is the degenerating fibroblast. (Fig 18)[11]

In 1997 de Waal J *et al* described buccal mucosal fibroblasts as belonging to three subpopulations namely F1 fibroblasts which are spindle shaped, highly proliferative and low collagen producing phenotype, F2 fibroblasts which are epitheloid, less proliferative and produce relatively higher amount of collagen and F3 fibroblasts that are stellate shaped, least proliferative and high collagen producing phenotype. They found a relative increase in the F3 cells when compared with F1 cells in OSF; this change was significantly larger than the controls.[10]

When the control group buccal mucosal fibroblasts subpopulations were analyzed for eight days, the last three days did not show any significant variation, hence the subpopulation was analyzed for only five days in both study and control groups. When the subpopulation were analyzed as F1,F2 and F3, we found a significant increase in F3 and F2 concentrations and a decrease in F1 concentration in both controls and study group. (Table 27) There was an increased trend in the F3 and F2 concentration in controls when compared to exposed group. Post mitotic fibroblasts were not observed in the exposed group when the subpopulations were analyzed using subpopulation model developed on human skin fibroblasts by Bayreuther *et al*[11]. This shows that there is an increased concentration of mitotic fibroblasts in exposed groups. The absence of post mitotic cells in the areca nut extract exposed cell lines could be because of the stimulation of cells by the areca alkaloids. The exposed extract contained 0.175µg/ml of arecoline. (Annexure I) This concentration of arecoline is found to increase the proliferation of fibroblast in earlier studies. Harvey W. *et al* (1996)[55] noted an increase in proliferation in fibroblast cells when exposed to arecoline and arecaidine of concentrations of 0.1µg/ml, 1 µg/ml and

10 µg/ml. J.H Jeng *et al* (1994)[33] had also showed a 20-40% increase in proliferation of cells in the presence of lime.

The increase in proliferation of fibroblasts in the exposed group may lead to exhaustion of mitotic potential and resulting in senescence and increased collagen production, and eventually leading to OSF.

In our study, fibroblast cells of patients without chewing habits showed an increased proliferation when exposed to areca nut product extract at a lower concentration when compared to the control group. This shows that areca nut exposure at very low concentrations on fibroblast can alter their cellular function, leading to pathology.

- The present study was undertaken to evaluate the effects of areca nut on cultured human buccal fibroblasts from subjects without areca nut chewing habits.
- Determination of F1:F2:F3 fibroblast subpopulation ratios, Mitotic: post mitotic fibroblast subpopulation ratios, growth curve with its derivatives were done to characterize the isolated fibroblasts.
- We estimated the concentration levels of arecoline and arecaidine in a commercially available areca nut product using HPLC.
- There was in an increase in post mitotic fibroblast and a decrease in mitotic fibroblast in the control group which was not statistically significant.
- Post mitotic cells were not observed on exposure to low concentration of areca nut extract (0.175µg/ml) in five days of observation.
- We found that there is an increase in F3 and F2 subpopulation ($p<0.001$) and a decrease in F1 subpopulation ($p<0.001$) within the control and study group.
- We found that there is an increased trend in F3 and F2 subpopulations of cells in controls when compared to the study group.

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Annexure I

- One sachet packet of commonly used commercial areca nut product contains 1.8gm
- Yield during extraction process for the areca nut product is 21% (Table 2)
- The amount of extract obtained from the areca nut product = 1.8 x 21 / 100

  = 0.378gm of extract
- Amount of arecoline estimated for the commercial areca nut product in deionozed water by HPLC
- 1µg/ml of the extract = 3.490µg/ml of arecoline

  = 0.00349 µg/µl of arecoline
- Percentage of arecoline in the extract = 0.00349 x 100 / 1

  = 0.3%
- One packet of commercial areca nut product = 1.8gm
- Extract from 1.8gm of product = 0.378gm
- Amount of arecoline in 0.378gm of extract = 0.378 x 0.3 / 100

  = 0.00013gm

  =1130µg of arecoline
- Amount of arecoine estimated for the commercial areca nut product in PBS whose pH adjusted to saliva (pH 7.8) = 11.638µg/ml

  = 0.011638 µg/µl of arecoline

  (Table 4, Graph 2)
- Percentage of arecoline from 1µg of extract in PBS

  = 0.011638 x 100 / 1

  = 1.16%

- One packet of commercial areca nut product = 1.8gm
- Extract from 1.8gm of product = 0.378gm
- Amount of arecoline in 0.378gm of extract in PBS = 0.378 x 1.16 / 100

  = 0.00438gm

  = 4380µg of arecoline
- The amount of arecoline leached out from 1.8gm of product (extract weight 0.378gm)

  = 0.00438gm of arecoline
- Percentage of arecoline leached out in deionized water = 0.3%
- The amount of extract to be added to the culture media to simulate saliva

  = 0.00438 / 0.3 x 100

  = 1.46gm
- Cells were exposed to 1µl of 1.46gm dissolved in 5ml media
- Amount of arecoline in 1ml media = 4380µg / 5

  = 875µg
- The amount of arecoline in 1 µl = 875 µg / 1000 = 0.875 µg

- 1µl was added to the 5ml media of a fibroblast culture plate
- Amount of arecoline in 1ml of media = 0.875 µg / 5ml

  = 0.175 µg/ml of arecoline

Figure 4: pH meter

Figure 5: Cyclo-mixer.

Figure 6: Electronic weighing balance.

Figure 7: Centrifuge.

Figure 1: Phase contrast microscope

Figure 2: CO_2 incubator.

Figure 3: Laminar flow.

Figure 8: -20 °C Freezer.

Figure 9: Hot air oven.

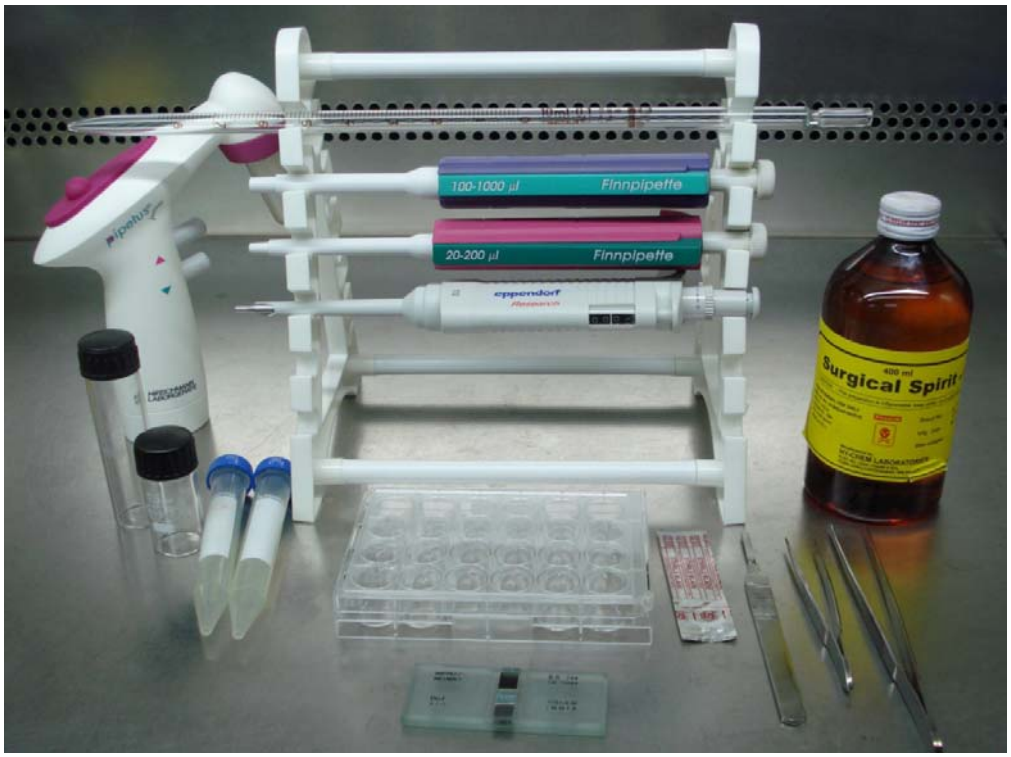

Figure 10: Cell culture armamentarium.

Figure 11: Cell culture reagents.

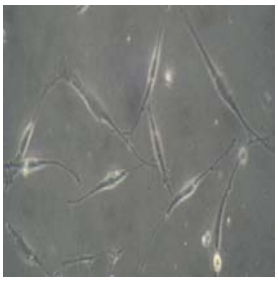

Figure 12

F1 fibroblast (20X)

Figure 13

F2 fibroblasts (20X)

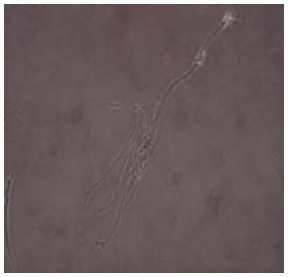

Figure 14

F3 fibroblasts (40X)

Figure 15

F4 fibroblasts (40X)

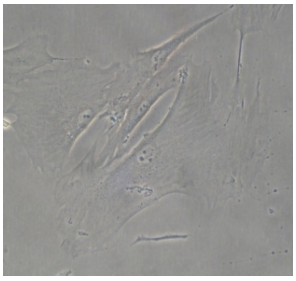

Figure16

F5 fibroblast (40X)

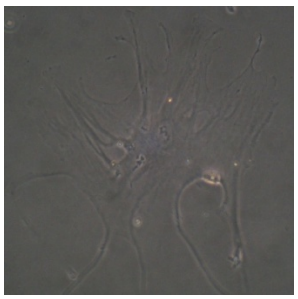

Figure 17

F6 fibroblast (40X)

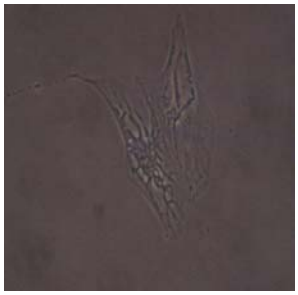

Figure 18

F7 fibroblast (40X)